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## **APPENDIX B**

the effect of the specific PI 3-kinase inhibitor wortmannin on STAT3 phosphorylation (Fig. 5). Cells were pretreated with various concentrations of wortmannin before addition of IFN- $\alpha$  and lysed. Proteins from lysates were precipitated with anti-pTyr and blotted with anti-STAT3 or anti-TYK2. Wortmannin produced a dose-dependent reduction in the slowly migrating band of STAT3, which is phosphorylated on both serine and tyrosine (median inhibitory concentration  $IC_{50} \approx 3$  nM). This was not attributable to inhibition of tyrosine phosphorylation events, because wortmannin had little or no effect on phosphorylation of the faster migrating band, which contains STAT3 solely phosphorylated on tyrosine, or of the TYK2 JAK kinase. Moreover, wortmannin (10 nM, 30 min) produced a ~50% reduction in ISG54 gene induction, consistent with the finding that serine phosphorylation of STAT3 is required for maximal activation of transcription (16). These findings indicate a role for the PI 3-kinase pathway in the serine phosphorylation of STAT3. It remains to be established whether PI 3-kinase mediates these events directly or indirectly through the IFN- $\alpha$ -activated PKCs, PKC $\delta$  and PKC $\epsilon$  (14, 17, 18, 22, 28).

Our results indicate that PI 3-kinase is coupled to the IFN-R through STAT3. Upon ligand-dependent tyrosine phosphorylation, residues Tyr<sup>527</sup> and Tyr<sup>538</sup> of IFNAR1 recruit STAT3 to the receptor; then, STAT3 itself undergoes tyrosine phosphorylation at residues Tyr<sup>656</sup> and Tyr<sup>705</sup>. Because both SH2 domains of p85 are required for the strongest interaction with the IFNAR1 signaling complex, two tyrosine-phosphorylated STAT3 molecules may be needed to dock p85 efficiently. Once docked, p85 can also undergo IFN-dependent tyrosine phosphorylation. Activated PI 3-kinase can then promote the serine phosphorylation of STAT3, which is critical for the formation of stable STAT3 homodimers or STAT3-STAT1 heterodimers (16, 29). These data add an important new facet to the role of STAT proteins in cellular signal transduction pathways.

## REFERENCES AND NOTES

- A. A. Branca, C. R. Faltynek, S. B. D'Alessandro, C. Baglioni, *J. Biol. Chem.* **257**, 13291 (1982).
- G. Uze, G. Lutfalla, I. Gresser, *Cell* **60**, 225 (1990).
- D. Novick, B. Cohen, M. Rubinstein, *ibid.* **77**, 391 (1994).
- P. Domanski et al., *J. Biol. Chem.* **270**, 21606 (1995).
- O. R. Colamonti, B. Porterfield, P. Domanski, S. N. Constantinescu, L. M. Pfeffer, *ibid.* **269**, 9598 (1994).
- S. N. Constantinescu et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9602 (1994).
- O. Colamonti et al., *Mol. Cell. Biol.* **14**, 8133 (1994).
- R. L. Friedman and G. R. Stark, *Nature* **314**, 637 (1985).
- A. C. Larner, A. Chaudhuri, J. E. Darnell Jr., *J. Biol. Chem.* **261**, 453 (1986).
- J. E. Darnell Jr., I. M. Kerr, G. R. Stark, *Science* **264**, 1415 (1994).
- C. Schindler, K. Shuai, V. R. Prezioso, J. E. Darnell Jr., *ibid.* **257**, 809 (1992).
- X.-Y. Fu, *Cell* **70**, 323 (1992).
- L. M. Pfeffer, B. Strulovici, A. R. Saltiel, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6537 (1990).
- L. M. Pfeffer et al., *ibid.* **88**, 7988 (1991).
- N. C. Reich and L. M. Pfeffer, *ibid.* **87**, 8761 (1990).
- Z. Wen, Z. Zhong, J. E. Darnell Jr., *Cell* **82**, 241 (1995).
- B. M. T. Burgering and P. J. Coffer, *Nature* **376**, 599 (1995).
- T. F. Franke et al., *Cell* **81**, 727 (1995).
- Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
- J. E. Mullersman and L. M. Pfeffer, *Trends Biochem. Sci.* **20**, 55 (1994).
- Z. Songyang et al., *Cell* **72**, 767 (1993).
- C. H. Yang et al., *J. Biol. Chem.* **271**, 8057 (1996).
- C. H. Yang and L. M. Pfeffer, unpublished data.
- R. Kapeller and L. C. Cantley, *Bioessays* **16**, 565 (1994).
- S. Uddin et al., *J. Biol. Chem.* **270**, 15938 (1995).
- N. Stahl et al., *Science* **267**, 1349 (1995).
- L. A. Goldman, E. C. Cutrone, S. V. Kotenko, C. D. Krause, J. A. Langer, *Biotechniques* **21**, 1013 (1996).
- A. Toker et al., *J. Biol. Chem.* **269**, 32358 (1994).
- X. Zhang, J. Blenis, H.-C. Li, C. Schindler, S. Chen-Kiang, *Science* **267**, 1990 (1995).
- S. N. Constantinescu et al., *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10487 (1995).
- H. Yan, K. Krishnan, J. T. E. Lim, L. G. Contillo, J. J. Krolewski, *Mol. Cell. Biol.* **16**, 2074 (1996).
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## Role of the Major Antigen of *Mycobacterium tuberculosis* in Cell Wall Biogenesis

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The dominant exported proteins and protective antigens of *Mycobacterium tuberculosis* are a triad of related gene products called the antigen 85 (Ag85) complex. Each has also been implicated in disease pathogenesis through its fibronectin-binding capacities. A carboxylesterase domain was found within the amino acid sequences of Ag85A, B, and C, and each protein acted as a mycolyltransferase involved in the final stages of mycobacterial cell wall assembly, as shown by direct enzyme assay and site-directed mutagenesis. Furthermore, the use of an antagonist (6-azido-6-deoxy- $\alpha,\alpha'$ -trehalose) of this activity demonstrates that these proteins are essential and potential targets for new antimycobacterial drugs.

**M**ycobacterium tuberculosis possesses a cell wall dominated by covalently linked mycolic acids, D-arabino-D-galactan, and peptidoglycan (mAGP), the mycolic acids of which are complemented by glycolipids such as  $\alpha,\alpha'$ -trehalose dimycolate (TDM, cord factor) and  $\alpha,\alpha'$ -trehalose monomycolate (TMM) (1). This mycolic acid-based permeability barrier shields the organism from environmental stress and contributes to disease persistence and the refractoriness of *M. tuberculosis* to many antibiotics (1). The success of chemotherapeutic agents such as isoniazid and ethambutol that specifically inhibit cell wall biogenesis confirms the necessity of this structure for bacterial survival (2). The biosynthetic pathways leading to formation of the key mycobacterial cell wall components, arabinogalactan (AG) and mycolic acids, are

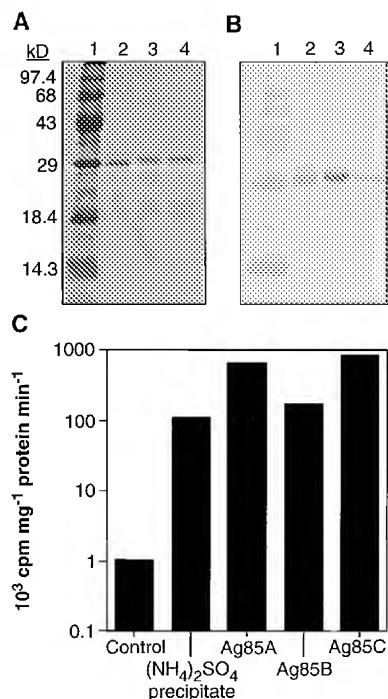
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therefore desirable targets for the rational design of new antituberculosis agents (3, 4). However, there is little information on individual enzymes (5, 6) or genes (7) involved in these unique processes.

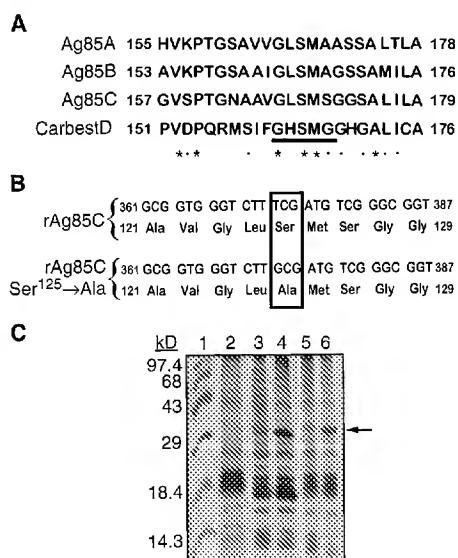
To define the enzymes and genes responsible for mycolic acid deposition, we developed a mycolyltransferase assay in which non-radioactive mycolic acids from lipid-soluble TMM were transesterified to radioactive water-soluble [ $^{14}\text{C}$ ] $\alpha,\alpha'$ -trehalose, resulting in the formation of lipid-soluble [ $^{14}\text{C}$ ]TMM and [ $^{14}\text{C}$ ]TDM (6). The enzyme responsible for this exchange from *M. smegmatis* was purified to near homogeneity by conventional means (6), and the transferase activity, assessed in terms of product formation (8), was determined to be  $1.89 \times 10^4 \text{ cpm mg}^{-1} \text{ protein min}^{-1}$ . Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) (9) of this enzyme preparation revealed the presence of two major proteins with identical isoelectric points of ~5.1 and relative molecular masses of 31 and 34 kD. Amino acid analysis of the NH<sub>2</sub>-terminus of both proteins yielded the sequence RPGLPVEY (10). Unexpectedly, this sequence was similar to that reported for



**Fig. 1.** Mycolyltransferase activity of purified *M. tuberculosis* Ag85 proteins. **(A)** Polyacrylamide gel electrophoresis (30) of Ag85 proteins purified by hydrophobic interaction chromatography. Lane 1, molecular size standards; lane 2, purified Ag85B; lane 3, Ag85C; and lane 4, Ag85A. **(B)** Protein immunoblot analysis of the purified Ag85 products with monoclonal antibody HYT-27 as the probe. Lane designation same as in (A). **(C)** Specific mycolyltransferase activities of the 40%  $(\text{NH}_4)_2\text{SO}_4$  precipitate of CFP and purified Ag85A, B, and C. The control assay contained the  $(\text{NH}_4)_2\text{SO}_4$ -precipitated proteins from CFP inactivated with  $\text{CHCl}_3$ . All enzymatic assays were done as described (8).

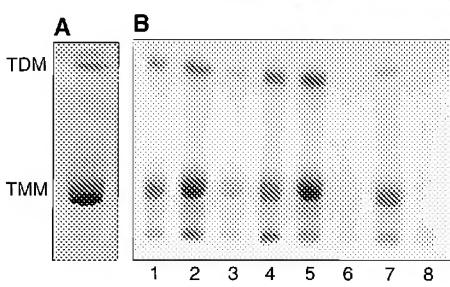
the A, B, and C components of *M. tuberculosis* Ag85 (FSRPGLPVEY) (11). Protein immunoblot analysis of the partially purified transferase from *M. smegmatis* showed that the 31- and 34-kD proteins were reactive to a monoclonal antibody (HYT-27) specific for Ag85 (12). These observations implicated members of the Ag85 complex in the exchange of mycolic acids within the mycobacterial cell wall.

The three closely related proteins (A, B, and C) of the *M. tuberculosis* Ag85 have been extensively characterized (13). Their fibronectin-binding capacities have led to concepts of involvement in complement receptor-mediated phagocytosis of *M. tuberculosis* (14) and the designation of their respective genes as *fba*, *fbb*, and *fbc* (15). However, the presence of Ag85 homologs in other non-pathogenic *Mycobacterium* spp. and in *Corynebacterium glutamicum* (13, 16) suggested a more fundamental, physiological role for these proteins. Thus, the individual components of the *M. tuberculosis* Ag85 complex were investi-



**Fig. 2.** Carboxylesterase consensus sequence within the Ag85A, B, and C proteins and its site-directed mutagenesis. **(A)** Alignment of partial amino acid sequences of the *M. tuberculosis* Ag85A, B, C, and the human carboxylesterase D by the Clustal program (31). Identical amino acids are indicated by an asterisk, and well-conserved amino acids by a dot. The carboxylesterase consensus sequence is underlined. **(B)** Partial sequences of the cloned *fbc* and the mutated *fbc* leaderless gene fragments. The boxed region shows the mutation to nucleotide 373 resulting in a Ser<sup>125</sup> to Ala mutation. **(C)** Polyacrylamide gel electrophoresis of whole cell lysates. Lane 1, molecular size standards; lane 2, *E. coli*:pET23b vector control; lane 3, *E. coli*:pCSB9 noninduced; lane 4, *E. coli*:pCSB9 IPTG-induced; lane 5, *E. coli*:pCSB9sa noninduced; and lane 6, *E. coli*:pCSB9sa IPTG-induced. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.

tigated for their role in TMM and TDM biogenesis. Culture filtrate proteins (CFPs), the source of the Ag85 components in the context of their antigenicity (13), from *M. tuberculosis* H37Ra were harvested from cells in mid-logarithmic growth (17) and precipitated with 40% saturated  $(\text{NH}_4)_2\text{SO}_4$ , yielding a fraction with substantial transferase activity (Fig. 1) and containing the full complement of Ag85 components as confirmed by protein immunoblot analysis. Full purification of the individual Ag85 proteins was achieved by hydrophobic interaction chromatography (Fig. 1A) (18). Protein immunoblot analysis verified that all were members of the Ag85 complex (Fig. 1B). Analysis by 2D PAGE (9) and silver nitrate staining (19) confirmed their purity and revealed migration patterns consistent with those previously reported (20). Mycolyltransferase activity measurement (8) of the individual proteins revealed that the Ag85A and Ag85C components had similar specific activities approximately six to eight



**Fig. 3.** Thin-layer chromatography and autoradiography of organic extractable products generated by the mycolyltransferase assay. TLC was done in a solvent system of  $\text{CHCl}_3:\text{CH}_3\text{OH}: \text{NH}_4\text{OH}$  (80:20:2) with silica gel TLC plates (Merck). **(A)** TDM and TMM standards were visualized by spraying with 10%  $\alpha$ -naphthol in 5% sulfuric acid in ethanol and heating at 110°C. **(B)** The  $\text{CHCl}_3$  organic extractable material from the mycolyltransferase reactions, in which the source of enzyme was as follows: lane 1, the 40%  $(\text{NH}_4)_2\text{SO}_4$  precipitate of CFP; lane 2, Ag85A; lane 3, Ag85B; lane 4, Ag85C; lane 5, a mixture of Ag85A, B, and C; lane 6, lysate from *E. coli*:pET23b; lane 7, lysate from *E. coli*:pCSB9; and lane 8, lysate from *E. coli*:pCSB9sa. The  $\text{CHCl}_3$  extract from each reaction mixture was dried and suspended in 100  $\mu\text{l}$  of  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1) of which 50  $\mu\text{l}$  was resolved by TLC. Products of these reactions were visualized by autoradiography.

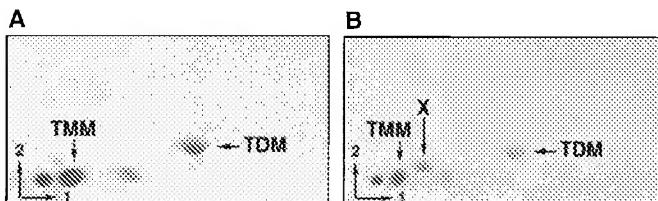
times greater than that of the initial preparation but that the specific activity of the Ag85B component was only about 20% of that for Ag85C (Fig. 1C).

Transesterification of mycolic acids as catalyzed by the Ag85 proteins dictates the necessity of carboxylesterase activity. Other fatty acyl transferases and lipases have a conserved carboxylesterase consensus sequence (Gly-Xaa-Ser-Xaa-Gly) (21), and x-ray crystallography of several carboxylesterases has defined the Ser residue as the active site of a catalytic triad consisting of Ser, Asp/Glu, and His (21). A search for functional domains within Ag85A, B, and C by amino acid sequence homology revealed a region in each, defined by amino acids 117 to 220 of Ag85A, having 34% homology to a 99-residue internal fragment of human carboxylesterase D (22) (Fig. 2A).

To confirm that the Ser residue of this putative active site was essential for transesterification of mycolic acids, a fragment of *fbc* encoding the leaderless *M. tuberculosis* Ag85C was obtained by polymerase chain reaction (PCR) amplification and ligated into the *Escherichia coli* expression vector pET23b, resulting in the recombinant plasmid pCSB9 (23). Site-directed mutagenesis of this cloned *fbc* gene fragment resulted in the replacement of Ser<sup>125</sup> with Ala (Fig. 2B); this plasmid was designated pCSB9sa (23). Transformation of *E. coli* BL21(DE3)pLysS with the pCSB9 and pCSB9sa, and induction with isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) re-

**Fig. 4.** Two-dimensional autoradiographic TLC of [ $1,2-^{14}\text{C}$ ]acetate pulse-labeled cells of *M. aurum* A<sup>+</sup> in the absence (**A**) and presence (**B**) of ADT. TLC plates were developed in the first dimension with  $\text{CHCl}_3:\text{CH}_3\text{OH}$ :

$\text{NH}_4\text{OH}$  (80:20:2) and in the second dimension with  $\text{CHCl}_3:\text{CH}_3\text{COOH}:\text{CH}_3\text{OH}:\text{H}_2\text{O}$  (50:60:2.5:3). Autoradiograms were obtained after exposure to Kodak X-Omat film at  $-70^\circ\text{C}$  for 12 hours.



sulted in the overproduction of a 32-kD protein by each recombinant clone, both of which reacted with the HYT-27 monoclonal antibody (Fig. 2C). Assay of whole cell lysates from these recombinant clones and *E. coli*:pET23b vector control demonstrated that only the induced *E. coli*:pCSB9 cells had appreciable activity ( $1.4 \times 10^4 \text{ cpm mg}^{-1} \text{ protein min}^{-1}$ ). Thin-layer chromatography (TLC) demonstrated that the products generated by native Ag85A, B, and C, and the recombinant Ag85C were true [ $^{14}\text{C}$ ]TMM and [ $^{14}\text{C}$ ]TDM (Fig. 3, A and B). However, the recombinant Ag85C with a Ser<sup>125</sup> to Ala mutation did not form these products or other acylated trehaloses (Fig. 3B), confirming the functionality of the carboxylesterase consensus sequence.

The abundance of TMM and TDM in the cell wall, as well as experimental data (24), indicate that these molecules are important to the integrity of the cellular envelope and that the TMM and TDM biosynthesis is a viable drug target. To substantiate this contention, several synthetic analogs of trehalose and TMM, putative competitive inhibitors of their metabolism, were assessed for inhibition of the growth of *M. aurum* A<sup>+</sup>, an established surrogate of *M. tuberculosis* for screening antituberculosis agents (25). One of these, 6-azido-6-deoxy- $\alpha,\alpha'$ -trehalose (ADT) (26), completely suppressed the growth of *M. aurum* but not of *E. coli* (minimal inhibitory concentration, 200  $\mu\text{g}/\text{ml}$  on solid media). Second, this compound at a concentration of 100  $\mu\text{g}/\text{ml}$  inhibited the in vitro mycolyltransferase activity of the purified recombinant Ag85C by ~60%. Third, analysis of the lipids and cell wall-bound (AG-containing) mycolic acids of [ $1,2-^{14}\text{C}$ ]acetate-labeled *M. aurum* (27), after treatment with a subinhibitory concentration of ADT (100  $\mu\text{g}/\text{ml}$ ), demonstrated marked inhibition of the synthesis of TMM (44 ± 7%), TDM (87 ± 5%), and cell wall-bound mycolic acids (62 ± 18%). Inhibition was accompanied by the accumulation of a new product (compound X, Fig. 4), an apparent intermediate in the mycolate exchange/transfer pathway. The sequence of events induced by ADT—that is, inhibition of the synthesis of TMM, TDM, cell wall-bound mycolates (and the emergence of an apparent intermediate), and of

cell growth—suggests that mycolate transfer or deposition, or both, are essential for bacterial viability, and the enzymes involved provide essential targets for the development of a new class of antimycobacterial chemotherapeutic agents directed against *M. tuberculosis*.

## REFERENCES AND NOTES

- P. J. Brennan and H. Nikaido, *Annu. Rev. Biochem.* **64**, 63 (1995).
- J. S. Blanchard, *ibid.* **65**, 215 (1996).
- G. S. Besra et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12735 (1994).
- B. A. Wolucka, M. R. McNeil, E. de Hoffmann, T. Chojnicki, P. J. Brennan, *J. Biol. Chem.* **269**, 23328 (1994); K. Mikusova, M. Mikus, G. S. Besra, I. Hancock, P. J. Brennan, *ibid.* **271**, 7820 (1996); R. E. Lee, K. Mikusova, P. J. Brennan, G. S. Besra, *J. Am. Chem. Soc.* **117**, 11829 (1995).
- A. Dessen, A. Quernard, J. S. Blanchard, W. R. Jacobs Jr., J. C. Sacchettini, *Science* **267**, 1638 (1995); A. Quernard et al., *Biochemistry* **34**, 8235 (1995).
- N. Sathyamoorthy and K. Takayama, *J. Biol. Chem.* **262**, 13417 (1987).
- Y. Yuan, R. E. Lee, G. S. Besra, J. T. Belisle, C. E. Barry III, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6630 (1995); K. M. George, Y. Yuan, D. R. Sherman, C. E. Barry III, *J. Biol. Chem.* **270**, 27292 (1995).
- Mycolyltransferase assays were performed by suspending 250  $\mu\text{g}$  of TMM in 450  $\mu\text{l}$  of 0.1 M potassium phosphate (pH 7.5), 0.1 mM dithiothreitol (DTT). To this was added 10 to 50  $\mu\text{g}$  of enzyme, and the mixture was sonicated for 30 s. [ $^{14}\text{C}$ ]Trehalose (20 nmol, 30.4 mCi/mmol) was then added, and the mixture was incubated at  $37^\circ\text{C}$  for 30 min. The reaction was stopped by adding 5 ml of  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1) and 1.4 ml of water. This mixture was vortexed and centrifuged, and the lower (organic) phase was collected and dried. This organic extractable material, containing the products of the mycolyltransferase reaction, was suspended in 100  $\mu\text{l}$  of  $\text{CHCl}_3:\text{CH}_3\text{OH}$  (2:1), and 50  $\mu\text{l}$  was added to 5 ml of scintillation fluid and counted.
- B. S. Dunbar, H. Kimura, T. M. Timmons, *Methods Enzymol.* **182**, 441 (1990).
- N. C. Price, Ed., *Protein Labfax* (Academic Press, San Diego, CA, 1996), p. 270.
- H. G. Wiker, K. Sletten, S. Nagai, M. Harboe, *Infect. Immun.* **58**, 272 (1990).
- S. Khanolkar-Young et al., *ibid.* **60**, 3925 (1992).
- H. G. Wiker and M. Harboe, *Microbiol. Rev.* **56**, 66 (1992); M. A. Horwitz, B. W. Lee, B. J. Dillon, G. Harth, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1530 (1995).
- P. Peake, A. Gooley, W. J. Britton, *Infect. Immun.* **61**, 4834 (1993).
- W. J. Philipp et al., *Proc. Natl. Acad. Sci. U.S.A.* **93**, 3137 (1996).
- G. Joliff et al., *Mol. Microbiol.* **6**, 2349 (1992).
- K. M. Dobos, K. Swiderlik, K.-H. Khoo, P. J. Brennan, J. T. Belisle, *Infect. Immun.* **63**, 2846 (1995).
- Protein obtained by precipitation with a 40% saturated solution of  $(\text{NH}_4)_2\text{SO}_4$  was dialyzed against storage buffer [10 mM  $\text{KH}_2\text{PO}_4$  (pH 7.5), 1 mM EDTA, 1 mM DTT], and 38 mg was applied to a column (1 cm by 10 cm) of Phenyl Sepharose (Pharmacia Biotech, Uppsala, Sweden). The col-
- umn was washed with three volumes of storage buffer at a flow rate of 1 ml/min, which eluted most of the proteins while leaving the Ag85 complex bound to the Phenyl Sepharose matrix. The individual proteins of the Ag85 complex were eluted with 30 ml of buffer A [10 mM tris-HCl (pH 8.6), 1 mM DTT, 1 mM EDTA] followed by a linear gradient composed of 100% buffer A to 100% buffer B [10 mM tris-HCl (pH 8.6), 1 mM DTT, 1 mM EDTA, 50% ethylene glycol] over a 40-ml volume followed by 10 ml of 100% buffer B.
- J. H. Morrissey, *Anal. Biochem.* **117**, 307 (1981).
- S. Nagai, H. G. Wiker, M. Harboe, M. Kinomoto, *Infect. Immun.* **59**, 372 (1991).
- R. J. Kazlauskas, *Trends Biotechnol.* **12**, 464 (1994).
- L. J. S. Young et al., *Hum. Genet.* **79**, 137 (1988).
- A 1.2-kb DNA fragment in pBluescript II SK- and containing *fbpC* was used as the template along with primers  $P_3$  (CATATGTTCTCTAGGCCGGCTT) and  $P_4$  (CTCGAGAGCCGGCCGGACGAGCAGG) to amplify by PCR the portion of *fbpC* encoding mature leaderless Ag85C. The resulting 0.9-kb DNA fragment was ligated into the Sma I site of pBluescript II SK-, amplified in *E. coli* DH5 $\alpha$ , isolated by digestion with *Nde*I and *Xba*I, and directionally ligated into the *E. coli* expression vector pET23b (Novagen, Madison, WI) to form a COOH-terminal His fusion product. This recombinant plasmid was designated pCSB9. Site-directed mutagenesis resulting in a T to G mutation of nucleotide 373 of the cloned *fbpC* fragment was accomplished by the PCR amplification of overlapping 390- and 531-base pair (bp) fragments with pCSB9 as the template. The 390-bp fragment was amplified with the primers  $P_3$  and  $P_5$  (5'-TCGCCTGACATCG[C]AA-GACCCACCGC-3'), a primer that overlaps nucleotide 373 and substitutes C for A at this position. The 531-bp fragment was generated with the primers  $P_4$  and  $P_6$  (5'-GCGGTGGGTCTT[G]CGATGTCGGGC-GGT-3'), which also overlapped with nucleotide 373 and substituted a G for T at this position. The overlapping 390- and 531-bp fragments were mixed, and a full-length 894-bp fragment was generated by overlap extension PCR (28) with the  $P_3$  and  $P_4$  primers. This product was ligated into pET23b, as described for the nonmutated *fbpC* fragment, to yield the recombinant plasmid pCSB9sa. Fidelity of the pCSB9 and pCSB9sa constructs was determined by sequence analysis.
- J. Liu, C. E. Barry III, G. S. Besra, H. N. Kaido, *J. Biol. Chem.* **47**, 29545 (1996).
- G. A. Chung, Z. Aktar, S. Jackson, K. Duncan, *Antimicrob. Agents. Chemother.* **39**, 2235 (1995).
- S. Hanessian and P. Lavallée, *J. Antibiotics* **25**, 683 (1972).
- Control and ADT-treated cultures were grown to an absorbance at 600 nm of ~0.2 before the addition of ADT (100  $\mu\text{g}/\text{ml}$ ) and preincubated at  $37^\circ\text{C}$  for 4 hours before the addition of [ $1,2-^{14}\text{C}$ ]acetate (Na salt; 110 mCi/mmol) at 1  $\mu\text{Ci}/\text{ml}$  to both control and ADT-treated cultures and further incubated with agitation for 20 hours at  $37^\circ\text{C}$ . Carbon-14-labeled lipids were isolated as described (29), and ~50,000 cpm of both control and ADT-treated extracts were applied to 2D TLC plates. Radioactive products resolved by 2D TLC were marked, extracted, and counted. The residual  $^{14}\text{C}$ -labeled, delipidated cells, rich in the bound mycolate esters of arabinogalactan, were subjected to alkaline hydrolysis, methylation, TLC, autoradiography, and counting (29).
- R. M. Horton, *Methods Mol. Biol.* **15**, 251 (1993).
- R. A. Slayden et al., *Antimicrob. Agents. Chemother.* **40**, 2813 (1996).
- U. K. Laemmli, *Nature* **227**, 680 (1970).
- D. G. Higgins, A. J. Bleasby, R. Fuchs, *Comput. Appl. Biosci.* **8**, 189 (1992).
- We thank M. Sonnenberg for technical support, M. Hein for preparation of the manuscript, and C. Mandander for graphics. Supported by grants AI-18357, AI-35220, and AI-38087 from the National Institute of Allergy and Infectious Diseases (NIAID), NIH, and SmithKline, Biotech, Uppsala, Sweden, and contract AI-25147 from NIAID.

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